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RESEARCH ARTICLE

The Effect of Dewandaru (*Eugenia uniflora L.*) Fruits Extracts on Testes Heat-induced Rats

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Abstract

Background: Spermatogenesis can run optimally if the testes temperature is 2-8^{o} C lower than body temperature. Higher testicular temperature causes oxidative stress, which results in infertility. The purple-red colour of Dewandaru (*Eugenia uniflora L.*) fruits have very high of flavonoid. The study aimed to examine the effect of Dewandaru (*Eugenia uniflora L.*) fruit extract on testis in heat-induced rats. Methods: The study was conducted at Integrated Biomedical Laboratory, Faculty of Medicine, Universitas Udayana between March until July 2019. This True experimental with randomised post-test only control group design consisted of a total of 24 rats. These were divided into four groups, with the group I and II was a negative and positive control. Group III and IV were treatment pre and post heat induction. Lipid peroxidation (MDA) level, Bcl-2, serum testosterone, and testis weight were measured. Data were presented as mean, and one-way ANOVA was used as a statistical tool with significant $p<0.05$. Result: Treatment with Dewandaru (*Eugenia uniflora L.*) fruit extract before and after heat induction, respectively showed a decrease significantly in the serum lipid peroxidase level, increase in the Bcl-2 expression, testosterone level, and testis weight. There is no significant difference between treatment pre and post heat induction. Conclusion: This study shows that Dewandaru (Eugenia uniflora L.) fruit extract altered the lipid peroxidase level, Bcl-2, testosterone level, and testis weight.

Keywords: *Bcl-2, Dewandaru fruits extract, Heat induction, MDA, Oxidative stress, Testosterone, Testis weight.*

Introduction

Infertility is a condition that a couple's inability to get pregnant after one or more years have regular sexual activity without protection or treatment [1]. Infertility affects almost 15% of couples on reproductive age. Male factors including the condition of decreased sperm quality cause 30-40% of infertility cases [2].

Various factors have been estimated to affect the decline in sperm quality, including physiological, environmental and genetic factors for oxidative stress (OS). An environmental factor that can influence in this case is work-related to radiation exposure and behaviour that can increase testicular temperature and testicular exposure to spermatotoxic substances [3]. Workers in the transportation industry have the highest risk of infertility compared to other jobs [4] as well as work-related to computers and sitting for long time also have 8 times higher risk of infertility [5]. Work that requires prolong sitting or exposure to heat can increase the testicular temperature which can cause disruption of spermatogenesis.

Spermatogenesis is a process that is affected by temperature and works optimally if the testicular temperature is 2-8^{o} C lower than body temperature [6]. Higher temperatures can cause an increase in testicular metabolism and have an impact on hypoxic

conditions that trigger oxidative stress [7,8]. Increased reactive oxygen species (ROS) or decreased levels of endogenous antioxidants can occur after heat induction. Increased testicular temperature cause DNA damage and lipid oxidation so that apoptosis occurs directly [6].

Increased testicular temperature also increases the production of ROS which can cause decreased sperm motility, increased sperm damage and decreased sperm-oocyte fusion ability [6,9]. Spermatogonia are very sensitive to the increase in ROS due to high polyunsaturated fatty acids (PUFA) in the plasma membrane. Various natural sources of antioxidants used to inhibit the effects of OS.

Phenolic derivatives, flavonoids, carotenoids, tocopherols and vitamin C found in natural materials have been believed to catch the ROS and metal chelating so that reaction of ROS with normal cell does not occur [10].

Dewandaru (*Eugenia uniflora L.*) is a shrub plant that has green colour fruit when young and purple-red when it is old. Dewandaru fruit is a source of ascorbic acid, anthocyanins, carotenoids and phenolics, especially flavonoids which have very high antioxidant activity [11,13]. In vitro, antioxidant activity of polyphenol compounds is very high in scavenging of ROS, nitrogen and chlorine species such as superoxide anions, hydroxyl radicals, peroxyl radicals, hypochlorous acids and peroxynitrite acids [13].

Dewandaru fruit extract dose of 200 mg/kg/day increase SOD and reduce MDA in rats with optimal physical exercise [14]. Increased SOD activity and decreased MDA levels indicate OS in the body has reduced through increased levels of endogenous antioxidant enzymes.

In this study, heat induction was given to the testes to obtain conditions of OS in the testicles. Furthermore, experimental rats were given the oral intake of dewandaru (*Eugenia uniflora L.*) fruit extracts to determine the effect in heat-induced rats. To find out the fruitfulness of dewandaru fruit extract as an antioxidant, MDA, Bcl-2, serum testosterone and testis weight were examined.

Materials and Methods

Study Design

This study is a true experimental study with a randomised post-test only control group design [15,16]. Twenty-four male Wistar rats (*Rattus norvegicus*) aged 2-3 months with 120-200 grams of weight originating from the laboratory of animal unit of the pharmacology and therapy department of the Medical Faculty Universitas Udayana were used in this study. The research began with the acclimation of experimental rats to the environment for one week. Rats are placed in cages containing 2 to 3 rats.

During the experimental period, rats had free access to food and water. Laboratory environment during the experiment period is clean, dry and good circulation conditions. This research has received ethical approval from the ethical committee of the Medical Faculty Universitas Udayana and Sanglah Central General Hospital.

Preparation of Dewandaru Fruit Extract

Dewandaru fruits with a purple-red colour were collected from Penglipuran Village, district of Bangli and validated by a taxonomist. Remove dewandaru seeds and cut into small pieces, then dried at 50^{o}C. Dry simplicia were mashed and soaked in 2000 ml of n-butanol solution for 3 days. Next maceration filtered and evaporated using a rotary evaporator and dried in water bath. Thirty grams of concentrated extract of dewandaru was obtained.

Animal Model

Rats were randomly divided into four groups. Namely group 1, negative control group (K-), rats were given immersion with the normal water temperature of 30^{o}C, 30 minutes a day for 6 days and CMC-Na by oral gavage 30 minutes before immersion. CMC-Na has given from the first day of immersion to 14 days after immersion (n = 6).

Group 2, positive control group (K +), rats were given immersion on heat water temperature of 40-43^{o}C 30 minutes a day for 6 days and CMC-Na by oral gavage 30 minutes before immersion. CMC-Na given from the first day of immersion to 14 days after immersion (n = 6).

Group 3, treatment group 1 (P1), rats were given immersion on heat water temperature 40-43°C 30 minutes a day for 6 days and dewandaru fruit extract with dose 200 mg/kg BW/day by oral gavage 30 minutes before immersion. Dewandaru fruit extract given from the first day of immersion to 14 days after immersion (n = 6).

Group 4, treatment group 2 (P2), rats were given dewandaru fruit extract with dose 200 mg/kg BW/day by oral gavage 7 days before immersion. Then rats given immersion at heat water temperature 40-43°C, 30 minutes a day for 6 days and dewandaru fruit extract 200 mg/kg/day given every day until 14 days after immersion in warm water (n = 6).

Testicular Heat Induction

Prepare a water bath with a temperature 40-43°C. Remove rat from the cage and put into a special immerse cage. Then the rats were immersed in the lower third of the rat (abdomen, hind legs to tail) into water for 30 minutes a day in 6 consecutive days. After 30 minutes, the rats were removed from the water and a special immerse cage and then dried. Check the scrotum for redness or injury. Rat that suffered injuries were excluded from the study. Rats that did not experience injury were returned to the maintenance cage [17].

Administering Dewandaru Fruit Extract

The rats were weighed first to determine the amount of daily extract to be given. Dissolve dewandaru fruit extract according to body weight using 1 ml of CMC-Na. Dewandaru fruit extract solution was given by oral gavage 30 minutes before immersion. Then, Rats were returned to the cage

Surgical Procedure and Sampling

After experimental periods, 15th day after immersion, all rats were given anaesthetics using a 10% ketamine dose of 40 mg/kg BW on the thigh. Furthermore, rats were taken blood samples and performed surgery to take testicular tissue. Furthermore, the testicular tissue immerses in a 10% formalin solution. After testicular tissue was removed, the rats were euthanasia administered by injecting a combination of Ketamine-Xylazine at a dose of 3 times the dose of anesthetics given intracardially.

Measurement of the Serum Level of MDA

Malondialdehyde (MDA) levels were measured using the MDA enzyme-linked immunosorbent assay (ELISA) kit (Elabscience). Protocol based on the assay kit used. Absorbance was measured at 450 nm.

Measurement of the Serum Level of Testosterone

Measurement of serum testosterone levels using the testosterone enzyme-linked immunosorbent assay (ELISA) kit (Bioassay Technology Laboratory, China). Protocol based on the assay kit used. Absorbance was measured at 450 nm.

Measurement of the Expression of BCL-2

Expression of BCL-2 measured using the immunohistochemical study. Pieces of 4-6 μm thick spermatogenic rat tissue were made on the object-glass. Tissue fragments are then processed for immunohistochemical staining as follows.

First, deparaffinization of xylol 1, for 10 minutes, xylol 2 for 5 minutes and absolute alcohol, 95% alcohol, 90%, 80% and 70% alcohol respectively for 5 minutes. Then the slides were dropped with PBS at pH 7, 4 for 10 minutes, and cleaned using tissue paper. Then the slides were dropped using EDTA for 20 minutes, and cleaned with tissue paper. Blocking with 1% BSA for 30 minutes and washing with PBS for 3x5 minutes, then cleaned with tissue paper.

Furthermore, it was dropped with primary antibody diluted with PBS and 0.1% twin solution in a ratio of 1:50. The preparation then incubated for 24 hours at 4°C, then washed with PBS pH 7.4 for 3x5 minutes and cleaned with tissue paper. The preparation is then dropped with a secondary antibody IgG biotin in PBS for 1 hour at room temperature.

The tissue then cleaned with tissue and added streptavidin Horse Radish Peroxidase (HRP) at room temperature for 30 minutes. The dosage is dropped by DAB for 20-40 minutes, then washed with distilled water for 3x5 minutes, and cleaned with tissue paper. Counterstained with meyer hematoxylin for 10 minutes, then washed and dried with air, then mounted with a lighter.

The brownish colour in histochemistry is the positive cell Bcl-2 protein [18].

Measurement of the Testis Weight

Testis weight measured using digital scale after the testes remove from the rat's body. Testis weight expressed in grams.

Statistical Analysis

Data were displayed as mean and standard error (SE). Data were analysed using Sapiro Wilk to determine the data distribution was normal or not. Furthermore, the data were analysed using one-way analysis of variance (ANOVA) followed by post hoc tests with Bonferroni test if the data was normal distribution and with Games-Howell test if the data was not in normal distribution. The level of significance was at $p<0.05$.

Results

The Serum Level of MDA

Serum level of MDA was significantly increased in the K + group compared to the K- group *(p <0.05)*. Also, MDA level in the P1 and P2 groups significantly decreased in compared with the K + group *(p <0.05)* (Table 1; Figure 2A).

Table 1: The Mean of the Serum Level of MDA, Serum Level of Testosterone, Expression of Bcl-2 and Testis Weight of Rats in The Experimental and Control Groups

Group	MDA (ng/mL)	Testosterone (nmol/L)	Bcl-2 (%)	Testis weight (gram)
K-	688.46 ± 42.62†	7.28 ± 0.42†	50.32 ± 0.77†	2.42 ± 0.13†
K+	1334.84 ± 132.82*†	3.44 ± 0.35*	19.17 ± 0.51*	1.20 ± 0.07*
P1	447.71 ± 63.77†	6.22 ± 0.18†	23.90 ± 1.14*†	2.22 ± 0.11†
P2	339.72 ± 54.34*†	6.18 ± 0.32†	37.79 ± 0.88*†	1.98 ± 0.03†

Note: The asterisk sign (*) shows significant difference with the K- group, and the symbol of † means the significant difference with the K+ group ($p<0.05$)

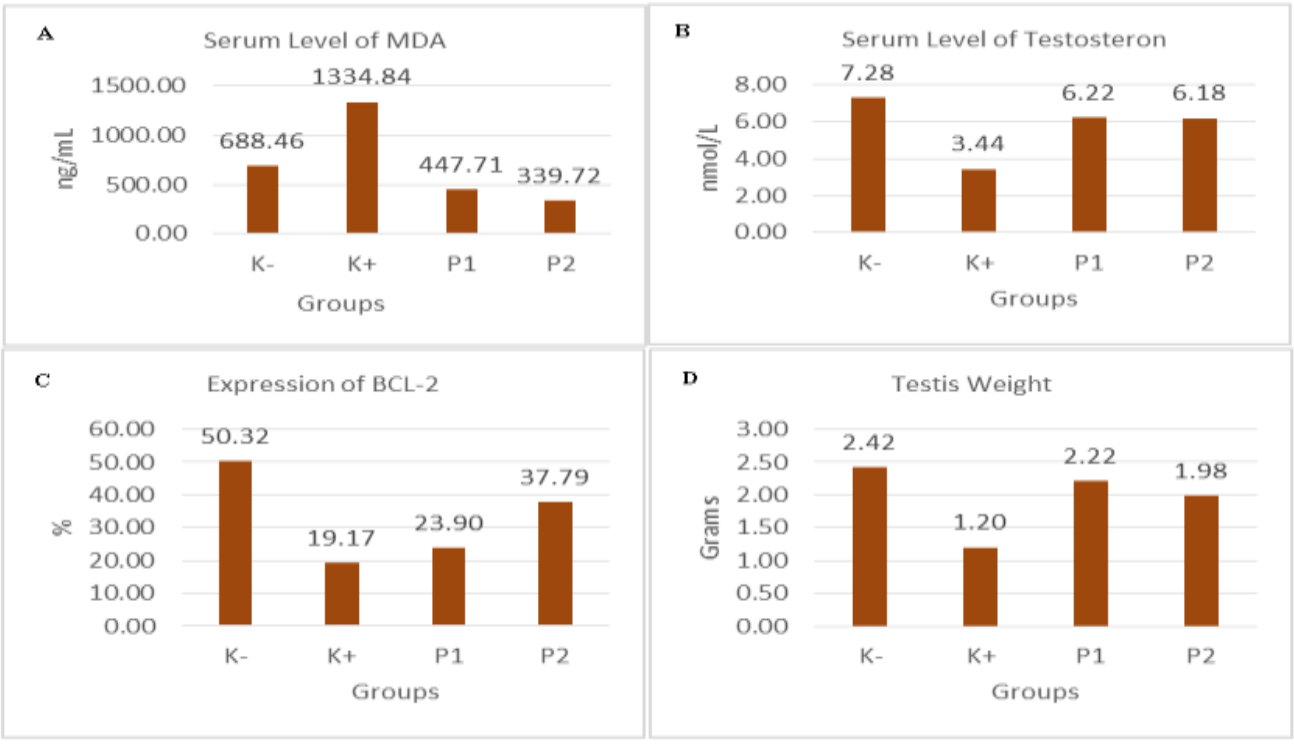

Figure 2: Comparison of the serum level of MDA (A), Testosterone (B), Expression of Bcl-2, (C) and Testis Weight (D)

The Serum Level of Testosterone

Serum level of testosterone was significantly decreased in the K + group compared to the K- group ($p<0.05$). Also, Testosterone level in the P1 and P2 groups increased dramatically in compared with the K + group ($p<0.05$) (Table 1; figure 2B).

Expression of BCL-2

Expression of BCL-2 was significantly decreased in the K + group compared to the K- group ($p <0.05$). Also, Expression of Bcl-2 in the P1 and P2 groups significantly increased in compared with the K + group ($p <0.05$) (Table 1; Figure 2C).

Testis Weight

Testis weight was significantly decreased in the K + group compared to the K- group *(*p <0.05*).* Also, Testis weight in the P1 and P2 groups was significantly increased in compared with the K + group (p <0.05) (Table 1; Figure 2C).

Discussion

Reactive oxygen species (ROS) are naturally produced by cells as a side product of aerobic metabolism [19]. Small amounts of ROS are needed in the normal function of spermatozoa for capacitation, hyperactivation, motility, acrosome reactions, oocyte fusion and fertilisation [7].

Increase in ROS harm sperm quality, namely decreased sperm motility, increased sperm damage and decreased oocyte-sperm fusion which results increase in men infertility [9]. This study found that testicular heating caused increased MDA levels, decreased testosterone levels, decreased Bcl-2 expression, and decreased on testicular weight. Oxidative stress occurs due to an increase in ROS production due to temperature increase in the testes. Increased testicular temperature causes an increase in cell metabolism which is not followed by an increase in oxygen supply resulting in high production of ROS [7,8,20].

ROS is a molecule that has free electrons and always reacts with other molecules to produce the other molecules with free electrons. ROS attacks PUFA (polyunsaturated fatty acids) in cell membranes, which can produce lipid peroxidation. Increased levels of lipid peroxidation (MDA) indicate oxidative stress [19].

Increased in testicular temperature, also responded by Bax (Bcl-2-associated X protein) by accumulating in the mitochondria and endoplasmic reticulum. The effect of this condition is Bcl-2 became localised to the mitochondrial membrane and phosphorylated so it becomes inactive [17]. The inactivity of Bcl-2 as an antiapoptotic factor causes the caspase reaction and increase in apoptosis on spermatogonia [21,22].

Increase in testicular temperature also increased in apoptosis of Leydig cells and caused decreased in testosterone production [23].

Increase in ROS production also causes damage to spermatogonia and other endocrine cells, causing disruption of hormonal balance [24,25]. Due to the high level of ROS, it activates the HPA axis (Hypothalamus-Pituitary-Adrenal axis) to secrete corticosterone in as a stress response.

This stress hormone has cross-communication between the HPG (Hypothalamus-Pituitary-Gonad) and the HPA axis, which has an impact on decreasing LH production by the anterior pituitary gland. Decrease in LH causes failure to stimulate Leydig cells to secrete the testosterone so that the level of the testosterone also decreases [26].

ROS in the testes causes an increase in germ cell apoptosis and a decrease in serum testosterone levels. Decrease in serum testosterone levels also contributes to increased apoptosis and germ cell damage. The impact of this condition is the decrease in germ cell masses and decrease on the testis weight [6,27]. In the experiment group that was given dewandaru (*Eugenia uniflora L.*) fruit extract 200 mg/kg BW/day there was a significantly decrease in MDA levels ($p<0.05$), a significant increase in the expression of Bcl-2 ($p<0.05$), Testosterone levels ($p<0.05$) and testis weight ($p<0.05$).

Decreased in the serum level of MDA because of dewandaru fruit extract has a high content of flavonoids, phenolic, terpenoids, and tannins. Phenolic compounds of the dewandaru fruit extract as a radical scavenger.

Phenolic compounds, chlorophyll, terpenoids contain many -OH groups, >C= C<, >C=O. This group able to donate one of its Hydrogen molecules to ROS thus becoming less reactive [28]. Flavonoids from natural herbs can protect PUFA on phospholipid membranes by donating one of its hydrogen ions (H+) to radical peroxidised lipid.

Polyphenol compounds such as flavonoids are multifunctional which can act with free radicals as reducing agents, free radical scavengers, metal chelating and forming singlets oxygen [10]. Dewandaru fruit extracts have previous research that the content of flavonoids can reduce oxidative stress and increase SOD activity in rats with excessive activity [29].

Flavonoids derived from natural herbs can reduce MDA levels in rats with a high-glucose diet [30]. Flavonoids from garlic (*Allium cepa*) can reduce MDA levels and increase SOD activity on Doxorubicin-induced cardiotoxicity in rats [31]. Decrease in the marker of oxidative stress in this study, followed by an increase in the other markers. As a result of decreased oxidative stress, increase in the secretion of the testosterone occurs, an increase in Bcl-2 activity and ultimately an increase in testicular weight.

The return of the normal function of testicular tissue in the process of spermatogenesis occurs due to administration of dewandaru fruit extract which contains many flavonoids as a natural antioxidant. The return of the testosterone levels and increased activity of Bcl-2 causes a decrease in apoptosis.

This is evidenced by a significant increase in testicular weight in the treatment group compared to the positive control group. Based on the results of this study, there were no significant differences between treatment group 1 and treatment group 2 for all markers studied. This proves that the use of dewandaru fruit extracts as antioxidants can be given as pre-treatment (prevention) and post-treatment (curative). In terms of the effectiveness of time and costs, giving dewandaru fruit extract as a curative (post-treatment) can first option because it requires shorter time and relatively less cost.

Conclusion

Dewandaru fruit extracts with a dose of 200 mg/kg BW/day in rats with testicular heat induction can restore spermatogenesis function by decreasing oxidative stress (MDA), increasing testosterone level, and increasing activity of Bcl-2. There were no significant differences in the administration of Dewandaru fruit extract before treatment (preventive) or after treatment (curative).

Nursing Implications

From this study, immersion in warm water periodically on long time can increase in the testes temperature. Higher testes temperature cause DNA damage, germ cells damage and finally this condition can cause men infertility. Natural antioxidants as a curative can be given to restore the function of spermatogenesis and increase fertility.

Ethical Statement

Current study protocols have been approved by Ethical Committee Faculty of Medicine Universitas Udayana and the animal model has been approved by Faculty Veterinary Medicine, Universitas Udayana. All research procedures are following the Declaration of Helsinki.

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